Biochimica et Biophysica Acta 1828 (2013) 602–613

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Myogenic differentiation and lipid-raft composition of L6 skeletal muscle cells are modulated by PUFAs

Anne Briolay [a], Rami Jaafar [b], Georges Nemoz [b], Laurence Bessueille [a,*]

[a] Université Lyon 1, CNRS, UMR 5246, Institut de Chimie et Biochimie Moléculaires et Supramoléculaires, IMBL, F-69622 Villeurbanne, France
[b] Université Lyon 1, INSERM U1060, CarMeN Laboratory, F-69600 Oullins, France

ARTICLE INFO

Article history:
Received 9 March 2012
Received in revised form 8 October 2012
Accepted 10 October 2012
Available online 16 October 2012

Keywords:
Cell membrane lipid
Fatty acid
Membrane microdomains
Myogenic differentiation
p70S6K

ABSTRACT

Lipid composition and fatty acid analysis of the major classes of membrane phospholipids were determined during myogenic differentiation of L6 skeletal muscle cells. The cholesterol to glycerophospholipids ratio decreased during differentiation, both in total (TM) and detergent-resistant membranes (DRM). Analyses of the membrane lipids showed that differentiation had a major impact on the molecular composition of glycerophospholipids. A significant decrease in the concentration of saturated fatty acids was detected in glycerophospholipid classes, and to a lesser extent in sphingolipids, while the concentration of 16:1n-7, 18:1n-7 and 18:1n-9 increased. At the same time, the concentration of long polyunsaturated fatty acid chains decreased in TM and DRM glycerophospholipids, resulting in a lower saturated to unsaturated fatty acid ratio in myotubes as compared to myoblasts. Interestingly, the observed n-3/n-6 ratio was lower in differentiated cell membranes. PUFA supplementation of L6 cells led to an increase in myogenic differentiation correlated to an incorporation of added PUFAs in TM and DRM glycerophospholipids. As expected after n-3 PUFA supplementation, the n-3/n-6 ratio was clearly increased in TM and, surprisingly, this was also the case in isolated DRM. n-3 and n-6 PUFAs significantly and time-dependently increased the phosphorylation of kinase p70S6K1 during myogenic differentiation, revealing the activation of the upstream kinase mTORC1, a major regulator of cell cycle and protein translation. In contrast, PUFAs did not affect the phosphorylation of the kinase Akt, another pivotal regulator of cell metabolism. These results suggest that PUFA supplementation modified the membrane lipid composition and affected the differentiation of L6 cells.

1. Introduction

Skeletal muscle formation or myogenesis is a complex and highly regulated process that results in the conversion of proliferating skeletal myoblasts into polynucleated myotubes expressing the contractile apparatus [1]. Differentiation of proliferating myoblasts begins with the sequential expression of myogenic regulatory factors, followed by the exit of these myoblasts from the cell cycle, the expression of the proteins of the contractile apparatus, and finally the formation of multinucleated myotubes by cell fusion [1–3]. Skeletal myogenesis is mediated by a network of signal transduction pathways that regulates withdrawal from the cell cycle and specific myogenesis. In recent years, several signalling pathways have emerged as important regulators of muscle formation and maintenance. Among them, the p38 mitogen-activated protein kinase (p38 MAPK) and the phosphoinositide-3-kinase (PI3K)/Akt pathways are thought to have key roles in the control of muscle gene expression and muscle protein synthesis during myogenic differentiation [4–6].

Besides these intracellular mechanisms governing cell differentiation, cell surface properties (determined by membrane composition) are also of primary importance. Lipids are responsible for the physicochemical properties of the membrane itself, largely determining its asymmetry, fluidity and plasticity, and its organization in domains. Over the past few years, the simple model of biological membranes has been modified to recognize that one of their most important features is their ability to locally form specialized domains with a different composition and physical properties from the rest of the plasma membrane [7,8]. Lipid rafts or detergent-resistant membranes (DRM) are dynamic structures within the cell membrane, which are enriched in both sphingolipids with highly saturated acyl chains and cholesterol [9]. It is well accepted that lipids from the cellular membrane can be remodeled by altering dietary fat intake, especially by modulating the content of n-3 or n-6 polyunsaturated fatty acids (PUFAs) [10–12]. The incorporation of these PUFAs in membrane lipids considerably affects the physical properties of the membrane, including fluidity, phase transition temperature and lipid-protein interactions [13,14]. The level of unsaturation and the length of the phospholipid fatty acyl chains, as well as the cholesterol to phospholipid ratio have been found to influence a number of important functions in skeletal muscle. For example, the fluidity of

* Corresponding author. Tel.: +33 4 26 23 44 00; fax: +33 4 72 43 15 57.
E-mail address: laurence.bessueille@univ-lyon1.fr (L. Bessueille).

0005-2736/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamem.2012.10.006

the myoblast membrane increases as its cholesterol level falls and as its unsaturated fatty acid content rises [15]. Decreased insulin sensitivity of muscle tissue has been associated with decreased concentrations of polyunsaturated fatty acids in phospholipids, raising the possibility that changes in the fatty-acid composition of membranes modulate the action of insulin [16]. Several studies have demonstrated the modulatory role of long-chain PUFAs within the lipid microenvironment of certain enzymes [17–19]. Furthermore, it was recently observed that fatty acids modulate the proliferation and differentiation of C2C12 or L6 skeletal muscle cells [20,21]. Since PUFAs are incorporated in cell membrane lipids, some of their biological effects could be due to their effects on membrane composition and function [10,22]. However, no systematic studies of their incorporation in total cell membranes and in the detergent-resistant membranes have been carried out in both myoblasts and myotubes, in relation with myogenic differentiation.

In the present study, we have analyzed the lipid composition of total membranes (TM) and DRM isolated from L6 myoblasts and myotubes. We observed that not only the TM, but also the isolated DRM of L6 cells undergo a modification of their lipid composition during differentiation. We then used fatty acid-supplemented culture media to modulate membrane lipid-composition and to investigate the effects associated with fatty acid changes during differentiation. In an effort to elucidate the molecular mechanisms through which fatty acids influence myogenesis, we finally evaluated the status of the Akt/mTORC1/S6K1 signalling pathway in differentiating L6 cells submitted to fatty acid supplementation. This pathway is a central regulator of cell metabolism, growth, proliferation and differentiation.

2. Materials and methods

2.1. Materials

Dulbecco's modified essential medium (DMEM), fetal bovine serum (FBS), and antibiotic solution were from Life Technologies. All other reagent-grade chemicals (AVP, BSA, PUFAs, horseradish peroxidase-conjugated anti-mouse IgG) were obtained from Sigma-Aldrich. Antibodies against myogenin (F5D clone) and myosin (MF20 clone) were obtained from the Developmental Studies Hybridoma Bank (DSHB) developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology, Iowa City, IA 52242. Anti-caveolin, anti-phospho-p70S6K and anti-Akt were from BD Biosciences, Alexafluor 488 goat anti-mouse IgG was from Invitrogen. Creatine kinase assay kit was purchased from Sobioda, cholesterol assay kit from Molecular probes and phospholipid assay kit from Biolabo.

2.2. Cell culture

Cells of the subclone C5 (L6-C5), a clone that had shown a significant capacity for differentiation [23], were used throughout this study. L6C5 rat skeletal myoblasts (obtained from Dr. F. Naro, University of Roma, Italy) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose and supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM glutamine, 20 mM HEPES, 100U/ml penicillin and 100 μg/ml streptomycin (growth medium, GM) at 37 °C in a 5% CO_2 humidified atmosphere. Cells were subcultured every 2–3 days. For differentiation, cells were seeded at the density of 3000/cm^2. 72 h after plating, cultures were washed twice with Dulbecco's-PBS (D-PBS) and differenciation was induced by reducing the serum level to 1% and adding 0.1 μM Arg8-vasopressin (differentiation medium, DM). In these conditions, L6C5 myoblasts differentiate in 2–4 days.

2.3. Fatty acid treatment

The effects of different fatty acids on myogenic differentiation of L6 cells were determined by allowing the cells to differentiate in differentiation media supplemented with either fatty-acid free BSA (150 μM) or one of the following albumin-bound fatty acids (20 μM): oleic acid (OA; 18:1 n-9), arachidonic acid (AA; 20:4 n-6), eicosapentaenoic acid (EPA; 20:5 n-3) or docosahexaenoic acid (DHA; 22:6 n-3).

The molar ratio of fatty acids to albumin was 1:7.5.

2.4. Immunofluorescence staining

Cells cultured in 12-well dishes were washed with D-PBS, followed by fixation for 10 min with 3.7% paraformaldehyde and subsequently permeabilized in D-PBS containing 0.1% Triton X-100 for 10 min at room temperature. After three one-minute washes with D-PBS, the cells were blocked with 1% bovine serum albumin in D-PBS for 30 min and then incubated for 1 h with monoclonal myogenin or anti-myosin primary antibodies diluted with D-PBS to 1:1000. After four additional washes with D-PBS, the cells were incubated for 1 h at room temperature in the dark with Alexafluor-coupled secondary antibodies diluted in a blocking solution to 1:1000. Finally, the cells were washed three times in D-PBS. Fluorescent images were captured by an Axiovert-Zeiss inverted microscope (Zeiss, France). As a morphological parameter of muscle differentiation, the myogenic index was defined as the number of nuclei residing in the cell containing three or more nuclei divided by total number of nuclei in stained cells. The distribution of nuclei in myoblasts and myotubes was measured by counting the nuclei in at least 10 different locations selected randomly.

2.5. Creatine kinase assay

The activity of creatine kinase (CK) was measured to biochemically assess myogenic differentiation. After appropriate treatments and at the end of the incubation period, the cells were collected and centrifuged for 10 min at 500 ×*g*. The pellet was resuspended in 20 mM Tris–HCl pH 6.7, 1 mM EDTA. After sonication the homogenate was either used immediately for CK assays or stored at −80 °C for future use. The protein content in the samples was measured using the Bio-Rad protein assay reagent. CK activity was measured using a spectrophotometric-based kit (Sobioda). Specific activity of CK was calculated after correction for total protein content and defined as μmol/min per milligram of protein (μmol/min/mg).

2.6. Preparation of membrane fraction and isolation of DRM

The cells were homogenized in ice-cold Tris–HCl 25 mM pH 7.5, 150 mM NaCl, 5 mM EDTA containing a protease-inhibitor cocktail (TNE) and disrupted in a Potter homogenizer (40 strokes). The homogenate was subjected to centrifugation (900 ×*g*, 10 min) at 4 °C to remove unbroken cells and nuclei, and the supernatant was saved. This post-nuclear supernatant was centrifuged for 45 min at 100,000 ×*g* to obtain the total membrane (TM) fraction (plasma membrane and cytoplasmic vesicular structures). To maintain a constant detergent-to-protein ratio in all the experiments, the pellet was resuspended in TNE buffer and submitted to a protein assay. The TM fraction was incubated with 1% Triton X-100 at 4 °C for 30 min (final protein-to-detergent ratio: 3). In all cases, the samples were then mixed with an equal volume of 89% sucrose in Tris–HCl 25 mM pH 7.5, 150 mM NaCl, placed at the bottom of linear 5–40% sucrose density gradients and fractionated by flotation (centrifugation at 150,000 ×*g* for 20 h at 4 °C). 1 ml fractions were collected from the bottom of the ultracentrifuge tubes and submitted to further analysis.

2.7. Lipid analyses

Total lipids were extracted from membrane or microdomain samples according to a modification of the method of Bligh and Dyer [24]. One ml of each sample was mixed with 3.75 ml methanol/chloroform (2/1, v/v), vigorously shaken for 1 min, incubated at room temperature for 30 min and centrifuged for 10 min at 2000 ×*g*. The pellet contained precipitated proteins which were discarded. Chloroform (1.25 ml) and water (1.25 ml) were subsequently added to the supernatant and phase separation was performed by centrifugation at 2000 ×*g* for 10 min. The lower organic phase containing the extracted lipids was collected and dried under a stream of N_2. Total lipids were re-dissolved in chloroform and either analyzed by thin layer chromatography (TLC) or used for various assays. Total phospholipids were assayed by the ammonium ferrothiocyanate method [25] and by using the Enzymatic Colorimetric kit from Wako Chemicals (Germany). The enzymatic Amplex Red Cholesterol Assay kit from Molecular probes was used to quantify cholesterol.

Major lipid classes were separated by TLC in one dimension on 60 silica-gel plates (Merck, 25 × 25 cm), using two-solvent systems. Lipids were quantitatively applied to TLC plates and development was carried out in ethyl acetate–propanol–chloroform–methanol–0.25% aqueous KCl (25:25:25:10:9, by vol.). After the solvent had reached 10 cm from the bottom of the plates, the latter were dried thoroughly and developed in hexane–diethyl ether–acetic acid (75:21:4, v/v/v) for an additional 6 cm migration. The plates were then dried and lipids were stained by dipping the TLC plates into a mixture of 10% cupric sulphate (w/v) and 8% phosphoric acid (v/v). After 10 min at 180 °C the plates were scanned from the point of origin to the solvent front by using a Camag II TLC scanning densitometer. Quantification was carried out by *in situ* densitometry with absolute amounts of the lipid classes determined from co-chromatographed standards [26] using the CATS software analysis package from Camag. Lipid standards (0.5 to 10 ng) were prepared by dissolving commercially available lipids (phosphatidic acid, PA; phosphatidylcholine, PC; phosphatidylethanolamine, PE; phosphatidylglycerol, PG; phosphatidylinositol, PI; phosphatidylserine, PS; cholesterol, CH; and sphingomyelin, SM from Sigma) in chloroform.

2.8. Fatty acid analysis by GC

Total lipids were separated by 2D high-performance TLC (2D-HPTLC) using a CAMAG horizontal developing chamber. The developing solvent used for the first dimension was chloroform–methanol–NH_4OH (65:25:5), whereas the second dimension was developed in chloroform–acetone–methanol–acetic acid–water (30:40:10:10:5). Lipids were then stained by spraying the plate with a solution of 0.02% (w/v) dichlorofluorescein in 95% ethanol. The spots corresponding to the poorly resolved glycerophospholipids phosphatidylserine (PS) and phosphatidylinositol (PI) or sphingolipids (SL) on one hand, and the spots corresponding to phosphatidylcholine (PC) or phosphatidylethanolamine (PE) on the other hand were scraped and transesterified at 100 °C for 90 min in 250 μl of toluene-methanol (40:60) and 250 μl of BF_3 10% in methanol. The reaction was stopped in ice by adding 1.5 ml of 10% K_2CO_3 (w/v), and fatty acid methyl esters were extracted with 2 ml of isooctane. The derivatives were analyzed by gas chromatography (GC) using an Agilent Technologies chromatograph (model 6890) fitted with a SolGel 1 ms dimethylpolysiloxane capillary column (60 mm by 0.2 mm [inner diameter], 0.25-μm film thickness; SGE Europe, Ltd., France). The oven temperature was set at 80 °C for 1.5 min and increased to 150 °C at 20 °C min^{-1} and then to 250 °C at 2 °C min^{-1}. The temperature was maintained at 250 °C for 10 min before returning to the initial conditions. Helium was used as the carrier gas at 1 ml min^{-1}. The temperatures of the split/splitless injector and the flame ionization detector were set at 230 and 280 °C respectively.

2.9. SDS/PAGE and immunoblotting

Cell lysates and membrane fractions from L6 myoblasts or myotubes (20 μg protein) were separated by SDS-PAGE, transferred onto Hybond-C membranes (Amersham Pharmacia Biotech), and subjected to Western blot analysis with different antibodies. Briefly, for the analysis of myosin and caveolin-3, the first antibody incubation (overnight at 4 °C) was carried out with a 1:2000 dilution of the mouse monoclonal antibodies (MF20 and clone 26 respectively). Second antibody incubation was carried out with a 1:2000 dilution of anti-mouse immunoglobulin G antibody conjugated to HRP (Sigma). Immunostained bands were detected by the ECL method (GE Healthcare). The same Hybond-C membrane was stripped to detach the anti-caveolin or anti-myosin antibody and was subjected to western blotting using a monoclonal anti-α-tubulin antibody.

2.10. Statistical analysis

Data are presented as mean ± standard error (SE). For statistical comparison, the *t*-test was employed using XLSTAT software. *p*-values under 0.05 were considered to be statistically significant.

3. Results

3.1. Effects of the differentiation on lipid and fatty acid composition of total membranes and DRM

Modulation of membrane lipid composition is known to have an effect on various cell signalling pathways. In order to investigate the relationship between lipid composition and myogenic differentiation, we first isolated TM and DRM from undifferentiated and differentiated L6 cells, and we assessed their lipid and fatty acid compositions. To allow comparison of the results obtained with different cell populations, TM were extracted with cold Triton X-100 at a constant detergent to membrane-protein ratio. The resulting detergent-resistant preparations were fractionated using sucrose-density-gradient sedimentation. The Triton X-100 sucrose-density fractions were analyzed for total protein and cholesterol content, and specific raft markers (Fig. 1). The distribution of total proteins showed that most of the membrane proteins were detergent soluble as they appeared solubilized in the high density fractions. In contrast, the sucrose-density-gradient fractions showed a narrow distribution of the activity of alkaline phosphatase, a glycosylphosphatidylinositol-anchored enzyme considered a reliable marker of DRM, with the entire activity recovered in the low-density fractions (centred around 20% sucrose), as insoluble material. Caveolin is another raft-associated protein located at the cytosolic face of the membrane, and anchored by acylation and membrane-spanning segments. Western blot analysis performed on sucrose-density fractions showed that the bulk of caveolin-1 was recovered in the DRM fractions of both myoblasts and myotubes. Concerning caveolin-3, as expected, a strong increase of its expression was observed in differentiated cells compared to undifferentiated cells. Caveolin-3 was detected in the low-density fractions corresponding to the DRM of differentiated L6 myotubes. It should be noted that the purification of DRM was not impaired by the different levels of caveolin-3 expression between myotubes and myoblasts. A bimodal cholesterol profile was observed in both cases, with one peak corresponding to the low-density fractions, and the remainder found in the high-density fractions at the bottom of the gradient. Furthermore, the location of DRM-containing fractions was confirmed by dot-blot analysis of the reliable lipid raft marker ganglioside GM1. GM1 was highly enriched in a single peak, in both myoblasts and myotubes, indicating that virtually all of this glycosphingolipid was associated to the low-density fractions. In contrast the transferrin receptor, a negative marker for DRM, was excluded from the low-density fractions and localized at the bottom

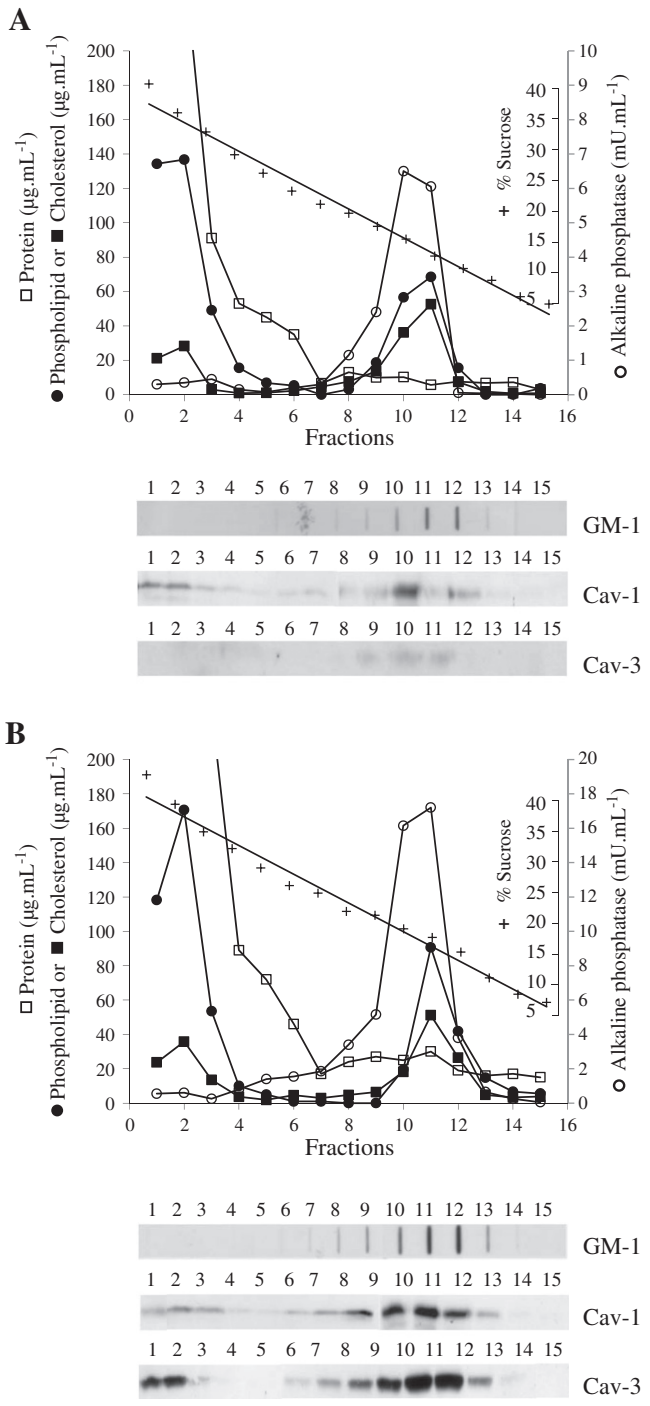

Fig. 1. Purification of DRMs. The total membranes of L6 myoblasts (A) or myotubes (B) were extracted with 1%Triton X-100 and fractionated by sucrose-density-gradient centrifugation. The distribution of phospholipids and cholesterol was determined in the gradient fractions collected from the bottom of the tube (Fraction 1: bottom of the gradient: 40% sucrose; fraction 15: top of the gradient: 5% sucrose). Phospholipid and cholesterol contents are expressed as µg/ml of fraction. The distribution of alkaline phosphatase, a GPI-linked DRM-associated protein, was examined by assaying the activity expressed in U mL^{-1}. Gradient fractions were analyzed by Dot-blotting or Western immunoblotting followed by ECL® detection for the lipid-raft markers GM1 and caveolins 1 and 3.

of the gradient, where the majority of the protein which had been solubilized by Triton X-100 was found (data not shown).

We then combined TLC, GC and enzymatic assays to compare the lipid composition of TM and DRM (Tables 1 and 2). In spite of a similar general profile, TM and DRM exhibited clear differences in terms of relative abundance of the different classes of lipids. Briefly,

Table 1
Relative proportions of different classes of lipids in the total membrane and DRM fractions (expressed as the molar ratio of the total lipid content analyzed for each sample).

	Myoblasts		Myotubes	
	TM	DRM	TM	DRM
Saturated/unsaturated	0.82 ± 0.20	1.28 ± 0.16#	0.66 ± 0.00*	1.16 ± 0.22#
n-3/n-6	0.621 ± 0.19	0.575 ± 0.15	0.322 ± 0.07*	0.316 ± 0.05
Cholesterol/GPL	0.326 ± 0.091	1.345 ± 0.162#	0.167 ± 0.064*	1.105 ± 0.050#
Sphingomyelin/GPL	0.123 ± 0.032	0.347 ± 0.073	0.125 ± 0.013	0.384 ± 0.047

These data represent the means from 3 separate experiments ± SE.
*$p<0.05$ myotubes vs. myoblasts #$p<0.05$ DRM vs. TM.

glycerophospholipids (GPL) in TM of myoblasts represented more than 50% of total lipids with major phospholipids being PC and PE. As expected, the DRM presented a much higher cholesterol to glycerophospholipid (CH/GPL) ratio. The differentiation of L6 cells was associated with changes of lipid class proportions. As shown in Table 1, differentiation induced a marked decrease in the CH/GPL in TM, from 0.326 in myoblasts to 0.167 in myotubes. The CH/GPL ratio was correlated with the ratio of saturated to unsaturated fatty acids (0.82 and 0.66 for myoblasts and myotubes respectively), as well as with the ratio of n-3 to n-6 fatty acids (0.621 and 0.322 for myoblasts and myotubes respectively). By contrast, the sphingomyelin to glycerophospholipid ratio observed in TM remained constant during the differentiation process.

Membrane sphingolipid and glycerophospholipid fatty acid compositions of L6 cells are shown in Table 2. When the relative molar composition (mol %) was considered, it was apparent that the predominant fatty acids in myoblast membranes were saturated (16:0, 18:0) and monounsaturated (18:1 n-9 and 18:1 n-7). PC mainly comprised saturated 16:0, while PE contained more 18:0. The higher contents of 16:0 in PC and of 18:0 in PE are consistent with the known predilection of these fatty acids in the sn-1 position of the corresponding glycerophospholipids. By comparison, SL contained much more 16:0 fatty acids, whereas the amount of 18:0 was similar. Myoblast membrane lipids contained 46% of saturated fatty acids on average, and the highest saturated/unsaturated ratio was observed in the sphingolipid fraction, followed by PI/PS, PC and finally PE. Furthermore, our results showed that glycerophospholipids were enriched in long-chain n-3 and n-6 series, as compared to sphingolipids. On the other hand, the presence of very long chain fatty acids, such as 24:0 and 24:1 n-9, was only observed in the SL fraction.

The greatest difference in myotube fatty acid content, as compared with myoblasts, was observed in the saturated fatty acids of all membrane lipids. Myotubes had a significantly reduced content of 16:0 and 18:0, with a concomitant increase in the monounsaturated fatty acids 16:1 n-7, 18:1 n-9 and 18:1 n-7. Thus, the ratio of saturated to unsaturated fatty acids was significantly lower in myotube membranes compared to those of myoblasts. On the other hand, the content in long chain polyunsaturated fatty acid (20:4 n-6; 20:5 n-3; 22:5 n-3 and 22:6 n-3) decreased during L6 differentiation. Differentiation was also accompanied by changes in the relative distribution of n-3 and n-6 polyunsaturated fatty acids. We observed that the ratio of n-3 to n-6 fatty acids in myotube membranes was significantly lower than in myoblast membranes (Table 1). In parallel, the total amounts of n-7 and n-9 fatty acids increased, with 18:1 n-7 being the most affected (Table 2).

We then determined whether DRM isolated from differentiated L6 myotubes showed differences in the relative proportion of phospholipid and in phospholipid-fatty acyl composition, as compared to the DRM from undifferentiated myoblasts. As mentioned above (Table 1) the CH/GPL ratio in TM was significantly higher in myoblasts than in myotubes. After extraction with 1% Triton X-100, the CH/GPL ratio in the DRM isolated from myoblasts was still higher

Table 2
Fatty acid composition of total membrane and DRM lipids from L6 myoblasts and myotubes.

	Myoblasts TM				Myotubes TM			
Fatty acids	SL	PC	PI/PS	PE	SL	PC	PI/PS	PE
14:0	0.48 ± 0.07	3.33 ± 1.72	0.17 ± 0.07	0.57 ± 0.09	1.42 ± 0.00	6.60 ± 0.98*	0.90 ± 0.01*	0.53 ± 0.03
16:0	51.84 ± 5.77	32.01 ± 5.32	5.32 ± 0.68	7.85 ± 1.29	57.21 ± 3.34*	25.09 ± 1.47*	5.08 ± 0.05	5.58 ± 0.31*
18:0	11.96 ± 2.62	10.88 ± 1.85	45.13 ± 2.84	18.5 ± 3.44	9.67 ± 0.40	7.22 ± 0.34*	35.89 ± 0.77**	10.62 ± 0.16*
20:0	2.58 ± 0.05	0.17 ± 0.08	0.4 ± 0.27	0.23 ± 0.22	1.43 ± 0.08***	0.12 ± 0.04	0.30 ± 0.08	0.32 ± 0.09
22:0	4.02 ± 0.87	0.05 ± 0.04	0.36 ± 0.17	0.21 ± 0.20	3.04 ± 0.31	0.03 ± 0.03	0.22 ± 0.09	0.15 ± 0.07
24:0	3.79 ± 1.97	0.04 ± 0.04	0.61 ± 0.25	0.78 ± 0.18	3.55 ± 0.51	0.00 ± 0.00	0.09 ± 0.04	0.02 ± 0.02
16:1 n-9	0.00 ± 0.00	3.33 ± 0.20	0.89 ± 0.01	0.68 ± 0.13	0.61 ± 0.04***	3.98 ± 0.02*	1.72 ± 0.22*	0.64 ± 0.17
16:1 n-7	0.00 ± 0.00	5.58 ± 0.36	1.63 ± 0.35	2.39 ± 0.52	0.22 ± 0.08*	13.58 ± 1.60*	5.85 ± 0.51***	7.02 ± 0.76***
18:1 n-9c	2.55 ± 1.07	20.18 ± 4.58	16.7 ± 3.07	17.37 ± 1.43	3.13 ± 1.92	20.33 ± 0.98	21.27 ± 0.81*	22.20 ± 0.54*
18:1 n-7	0.34 ± 0.29	11.35 ± 1.38	7.4 ± 0.32	5.63 ± 0.61	1.52 ± 0.52	15.68 ± 0.22*	14.03 ± 1.46*	12.08 ± 0.89**
18:2 n-6c	0.61 ± 0.25	2.83 ± 0.88	1.82 ± 0.23	2.06 ± 0.35	0.56 ± 0.24	0.68 ± 0.02*	0.48 ± 0.03**	0.73 ± 0.03*
20:2 n-6	0.00 ± 0.00	0.15 ± 0.22	0.23 ± 0.07	0.08 ± 0.04	0.12 ± 0.07	0.21 ± 0.13	0.05 ± 0.03	0.04 ± 0.01
20:3 n-6	0.24 ± 0.14	0.59 ± 0.13	1.54 ± 0.05	0.90 ± 0.13	1.79 ± 0.71	0.18 ± 0.00*	0.61 ± 0.02***	0.45 ± 0.01**
20:4 n-6	0.00 ± 0.00	2.72 ± 1.18	7.66 ± 1.92	10.82 ± 1.52	0.50 ± 0.11	0.51 ± 0.14	2.36 ± 0.04*	5.13 ± 0.53***
20:5 n-3	0.00 ± 0.00	0.58 ± 0.25	0.44 ± 0.02	2.33 ± 1.01	0.00 ± 0.00	0.04 ± 0.03*	0.06 ± 0.03*	0.67 ± 0.02
22:4 n-6	0.62 ± 0.47	0.09 ± 0.03	0.50 ± 0.21	1.21 ± 1.03	0.00 ± 0.00	0.03 ± 0.02	0.25 ± 0.00	0.78 ± 0.09
22:5 n-3	0.00 ± 0.00	0.69 ± 0.07	2.09 ± 1.03	5.31 ± 0.23	0.00 ± 0.00	0.14 ± 0.01***	0.74 ± 0.03	2.15 ± 0.10***
22:6 n-3	0.00 ± 0.00	0.54 ± 0.02	1.3 ± 0.31	4.38 ± 1.33	0.00 ± 0.00	0.16 ± 0.04***	0.50 ± 0.01	1.51 ± 0.13*
24:1 n-9	15.5 ± 1.22	0.05 ± 0.05	0.96 ± 0.49	0.05 ± 0.03	12.18 ± 0.68*	0.06 ± 0.03	0.11 ± 0.02	0.00 ± 0.00
Saturated/unsaturated	3.76 ± 0.87	0.96 ± 0.18	1.2 ± 0.17	0.53 ± 0.09	3.70 ± 0.05	0.70 ± 0.03*	0.95 ± 0.04	0.32 ± 0.01*
n-3/n-6	0.00 ± 0.00	0.28 ± 0.05	0.33 ± 0.13	0.80 ± 0.18	0.00 ± 0.00	0.21 ± 0.01*	0.35 ± 0.01	0.61 ± 0.05*
	Myoblasts DRM				Myotubes DRM			
Fatty acids	SL	PC	PI/PS	PE	SL	PC	PI/PS	PE
14:0	1.02 ± 0.45	5.14 ± 1.76	1.28 ± 1.01	1.37 ± 1.06	1.00 ± 0.04	7.96 ± 2.98	1.11 ± 0.35	0.95 ± 0.44
16:0	59.92 ± 9.32	42.51 ± 0.51#	9.85 ± 0.34##	11.37 ± 2.92	54.02 ± 6.96	38.02 ± 4.01#	14.73 ± 0.54*** ###	10.62 ± 1.12#
18:0	11.77 ± 2.46	9.57 ± 0.49	43.13 ± 2.01#	20.58 ± 1.73	10.86 ± 0.55#	7.86 ± 0.73	38.86 ± 2.71	13.66 ± 0.28* ###
20:0	2.26 ± 0.44	0.00 ± 0.00#	0.17 ± 0.14###	0.08 ± 0.04#	1.59 ± 0.42	0.07 ± 0.06	0.15 ± 0.13	0.16 ± 0.12
22:0	2.83 ± 0.92	0.00 ± 0.00	0.15 ± 0.12##	0.00 ± 0.00	2.85 ± 0.87	0.00 ± 0.00	0.00 ± 0.00#	0.03 ± 0.03
24:0	1.09 ± 0.54	0.00 ± 0.00	0.29 ± 0.24	0.55 ± 0.38	2.86 ± 0.95	0.01 ± 0.02	0.07 ± 0.06	0.01 ± 0.01
16:1 n-9	0.00 ± 0.00	2.50 ± 0.31###	1.18 ± 0.49	0.74 ± 0.16	0.49 ± 0.16*	2.53 ± 0.29##	1.51 ± 0.20	0.68 ± 0.15
16:1 n-7	0.00 ± 0.00	3.49 ± 0.59###	1.35 ± 0.06	1.57 ± 0.46###	0.42 ± 0.28	7.23 ± 2.01* #	3.21 ± 0.85#	4.04 ± 1.38*
18:1 n-9c	2.42 ± 0.62	15.7 ± 3.58	16.27 ± 3.38	16.81 ± 4.51	2.92 ± 1.29	15.57 ± 3.03	18.18 ± 2.48	20.39 ± 4.53
18:1 n-7	0.30 ± 0.24	10.19 ± 2.72	7.56 ± 0.84	6.24 ± 0.94	0.65 ± 0.34	12.27 ± 1.98	11.78 ± 1.86*	9.56 ± 2.15
18:2 n-6c	0.00 ± 0.00#	2.67 ± 1.08	2.31 ± 1.17	2.28 ± 0.67	0.91 ± 0.58	1.38 ± 1.01	1.19 ± 0.52	1.27 ± 0.70
20:2 n-6	0.00 ± 0.00	0.30 ± 0.07	0.99 ± 0.29#	0.68 ± 0.07	0.05 ± 0.04	0.08 ± 0.07	0.51 ± 0.48	0.68 ± 0.18
20:3 n-6	0.00 ± 0.00	0.22 ± 0.04#	1.13 ± 0.01###	0.78 ± 0.09#	0.05 ± 0.04	0.13 ± 0.07	0.39 ± 0.29	0.48 ± 0.18
20:4 n-6	0.30 ± 0.24	1.19 ± 0.77	5.44 ± 2.24	8.22 ± 1.87	0.45 ± 0.23	0.26 ± 0.19	1.51 ± 0.90	4.44 ± 2.54
20:5 n-3	0.42 ± 0.39	0.14 ± 0.07	0.00 ± 0.00###	1.15 ± 0.07	0.52 ± 0.39	0.05 ± 0.04	0.10 ± 0.10	0.7 ± 0.39
22:4 n-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00#	0.08 ± 0.02	0.06 ± 0.02	0.01 ± 0.01	0.08 ± 0.07	0.38 ± 0.19
22:5 n-3	0.00 ± 0.00	0.26 ± 0.09#	1.79 ± 0.23	4.24 ± 0.62#	0.21 ± 0.12	0.03 ± 0.03* #	0.39 ± 0.21*	1.60 ± 0.38*
22:6 n-3	0.00 ± 0.00	0.15 ± 0.02###	1.43 ± 0.13	3.54 ± 0.22	0.14 ± 0.09	0.04 ± 0.04* ###	0.26 ± 0.07*** #	1.01 ± 0.26**
24:1 n-9	11.08 ± 3.09	0.00 ± 0.00	0.22 ± 0.13	0.00 ± 0.00	13.22 ± 0.44#	0.03 ± 0.02	0.14 ± 0.12	0.00 ± 0.00
Saturated/unsaturated	5.44 ± 1.97	1.55 ± 0.20#	1.38 ± 0.06	0.73 ± 0.07#	3.64 ± 1.09*	1.36 ± 0.31#	1.40 ± 0.11#	0.56 ± 0.07* #
n-3/n-6	1.41 ± 0.00	0.13 ± 0.01#	0.33 ± 0.16	0.74 ± 0.17	0.58 ± 0.23	0.06 ± 0.06#	0.21 ± 0.11	0.46 ± 0.13

Fatty acid contents were expressed in mol% and the values represent the mean ± SE from three independent determinations.
*$p<0.05$, **$p<0.01$, ***$p<0.005$ myotubes vs. myoblasts.
#$p<0.05$, ##$p<0.01$, ###$p<0.005$ DRM vs. TM.

than in those from myotubes (1.345 and 1.105, respectively) (Table 1). It is noteworthy that the important increase of the CH/GPL and SM/GPL ratios in DRM as compared to TM is in accordance with the particular lipid composition of these domains, as commonly reported [9]. As observed for TM, the ratio of sphingomyelin to glycerophospholipids did not differ significantly between myoblast and myotube DRMs. The fatty acid compositions of sphingolipids and glycerophospholipids from L6 myoblast and myotube DRMs are shown in Table 2. As already observed for TM, the saturated to unsaturated ratio was decreased in DRM from differentiated cells compared to myoblasts. This decrease was essentially due to higher amounts of 16:1 n-7, 18:1 n-9 and 18:1 n-7 and lower percentages of saturated fatty acids in DRM from myotubes. The highest difference in fatty acid content between the two types of cells was observed in the saturated to unsaturated ratio in PC and PE species (Table 2). A relative decrease of long chain polyunsaturated fatty acids was also noticed. In regard to these PUFAs, DRM from myotubes had the lowest percentages of 20:4 n-6, 22:4 n-6, and 22:5 n-3. The total ratio of n-3 to n-6 PUFAs was markedly lower in DRM isolated from differentiated myotubes. These observations indicate that significant changes in DRM lipid composition occur during myogenic differentiation.

3.2. PUFA treatments affected proliferation and myogenic differentiation of L6 cells

Morphological and biochemical studies were performed to examine the effect of PUFAs on cell differentiation. Confluent cultures of L6 cells were incubated in differentiation medium in the presence of vehicle (i.e. fatty acid-free BSA) or 20 μM fatty acids (in complex with BSA) for 4 days. The morphology of each specimen was first assessed using phase microscopy. Upon visual inspection (Fig. 2A–E), the formation of myotubes was found to be significantly increased in the presence of 20 μM AA or DHA, and to a lesser extent of 20 μM EPA compared to the control cells. The cells were also examined for myogenin immunofluorescence to assess differentiation. Myotubes presented a characteristic organization with a large amount of myogenin-positive nuclei. PUFA-treated cells formed larger myotubes, with a higher number of

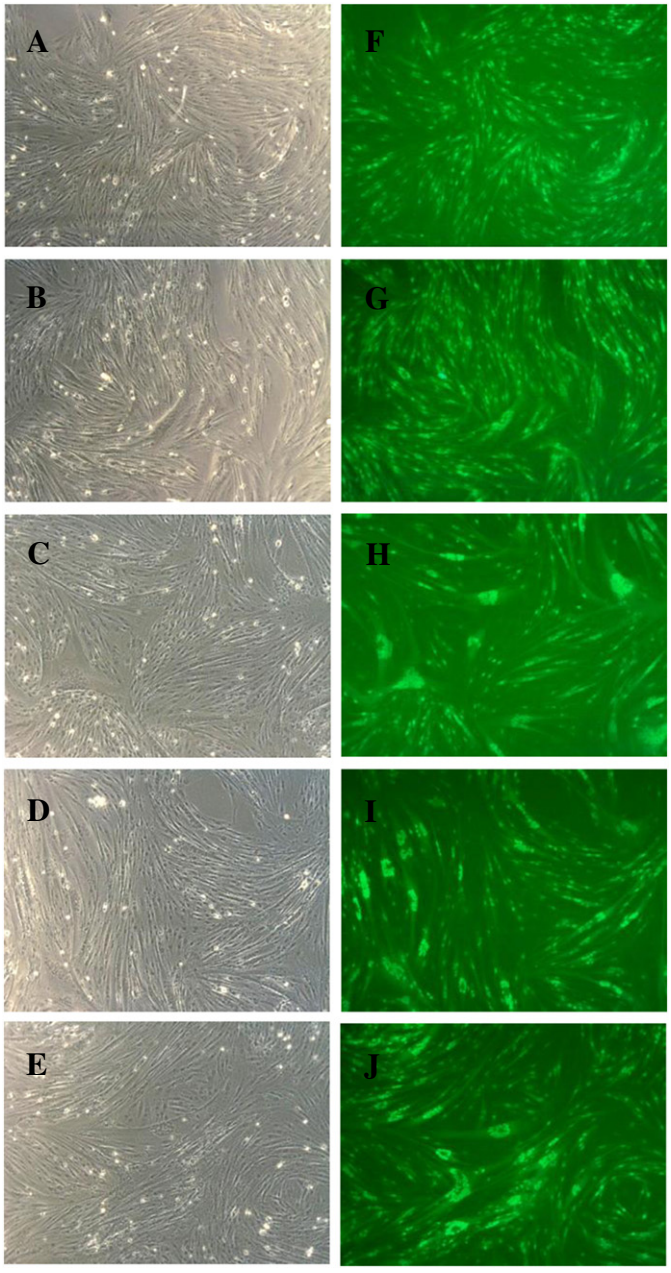

Fig. 2. Effect of PUFAs on myotube formation and myogenin expression. L6 cells were induced to differentiate in DM in the absence (control, A and F) or presence of 20 μM OA (B and G), AA (C and H), EPA (D and I), or DHA (E and J). Representative phase-contrast photographs of L6 cells after 4 days of treatment (A-E) and representative images of the myogenin immunofluorescence labelling (F–J). Nuclear myogenin was revealed by using a monoclonal anti-myogenin antibody and an Alexafluor 488-conjugated secondary antibody.

myotubes containing more than 20 nuclei than the control or OA-treated cells (Fig. 2F–J). This was confirmed by the quantification of the myogenic index, i.e. the fraction of nuclei residing in cells containing ≥3 nuclei, as a measure of myoblast fusion. In control cells, 27.7% of the nuclei were present in the differentiated cells after 96 h (Fig. 3). Both AA and DHA significantly enhanced L6 differentiation (Fig. 2H and J, Fig. 3) by increasing the myogenic index to 32.3% for 20 μM AA and 41.6% for 20 μM DHA. In parallel experiments, L6 myoblasts in proliferation medium were treated with the same concentrations of PUFAs during the same period of time. We observed a significant positive effect of all the tested PUFAs on myoblast proliferation, with the number of cells being increased by 20% in the presence of 20 μM AA and 30% with DHA (data not shown). Thus,

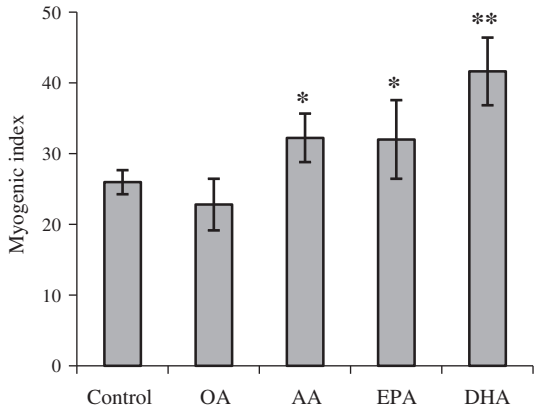

Fig. 3. Effect of PUFAs on the myogenic index. L6 cells were induced to differentiate in DM in the absence (control) or presence of 20 μM OA, AA, EPA or DHA for 4 days. The total number of nuclei and the number of nuclei incorporated into myotubes were counted. The myogenic index was defined as the fraction of nuclei residing in cells containing three or more nuclei. Data were expressed as the means ± SE of ten randomly chosen fields from triplicate samples. $^{*}p<0.05$ versus control, $^{**}p<0.005$ versus control.

PUFA treatments interfered with both myoblast proliferation and differentiation, as reflected by the increase in myotube formation.

To evaluate the ability of PUFAs to elicit the expression of muscle differentiation markers, we analyzed myosin and caveolin-3 contents, both known to be specific late markers of differentiation. Immunoblotting analysis, performed over a 4-day period, revealed that EPA and DHA greatly increased the expression of caveolin-3 (Fig. 4A). EPA- or DHA-treated cells showed about a 2.5-times higher expression of caveolin-3 with respect to control cells. Cells treated with 20 μM AA

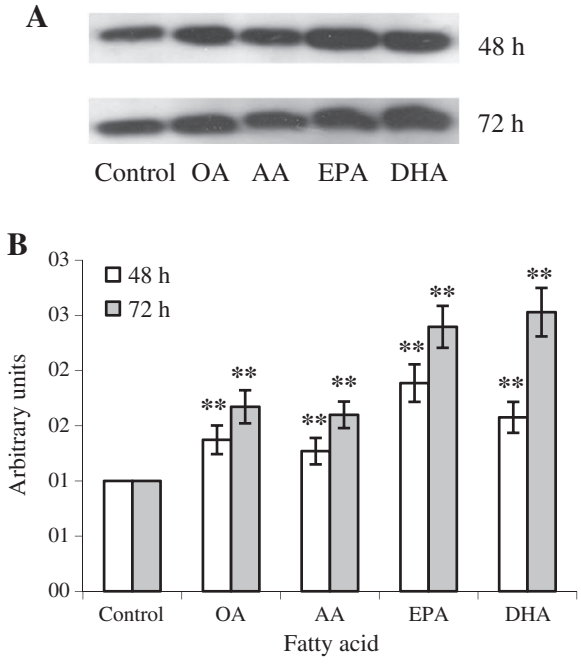

Fig. 4. Effects of PUFAs on the expression of caveolin-3. Caveolin-3 expression was measured by Western blot analysis of proteins extracted from L6 cells. At time 0 cells were shifted to DM in the absence (control BSA) or in the presence of 20 μM fatty acid and cultured for a further 48 or 72 h before solubilization and Western blotting with anti-caveolin antibody. (A) Representative Western blots of caveolin-3. 10 μg of proteins were loaded in each lane. (B) Quantitation of caveolin-3 expression in Western blots by densitometric analysis. The values were normalized to tubulin levels. Caveolin-3 amounts are expressed in arbitrary units. The graph shows the means ± SE of three different experiments. $^{**}p<0.005$ versus control.

showed a lesser increase in caveolin-3 expression (Fig. 4B). The expression of myosin was also increased in the presence of PUFAs, but to a lesser extent than caveolin-3 (Fig. 5).

We then assessed the activity of creatine kinase, which is generally accepted as a biochemical indicator of muscle-specific late gene expression during myogenesis. As expected, the activity of creatine kinase strongly increased (3 to 4-fold) in the presence of control differentiation medium, when compared with the baseline level of undifferentiated cells. When L6 myoblasts were treated with either the mono unsaturated fatty acid oleate or PUFAs, no significant difference was observed in the level of creatine kinase-specific activity (Fig. 6).

3.3. PUFA treatments modified the fatty acid composition of myotube membranes

Various studies have shown that PUFA treatments alter membrane lipid composition, causing functional effects on cell signalling [17–19]. Hence, we investigated whether exogenous PUFAs were incorporated into the L6 myotube membranes, leading to altered membrane lipid composition. The fatty acid composition of the membrane lipids of control myotubes, and myotubes differentiated in the presence of 20 μM monounsaturated OA (18:1n-9), polyunsaturated AA (20:4 n-6) or DHA (22:6 n-3), was analyzed (Table 3). When L6 cells were treated with OA or AA, the corresponding fatty acid and its elongation product (20:1 n-9, 22:1 n-9 and to a lesser extent 24:1 n-9, and 22:4 n-6, respectively) were enriched not only in the GPL of the TM but even more in isolated DRM when compared to control cells. The incorporation of the n-6 fatty acid AA greatly reduced the n-3 to n-6 ratio in TM and DRM, although no increase in the amount of n-6 PUFA was observed in sphingolipids. The incorporation of exogenous DHA was associated with an increased amount of both the supplemented fatty acid and EPA (20:5 n-3).

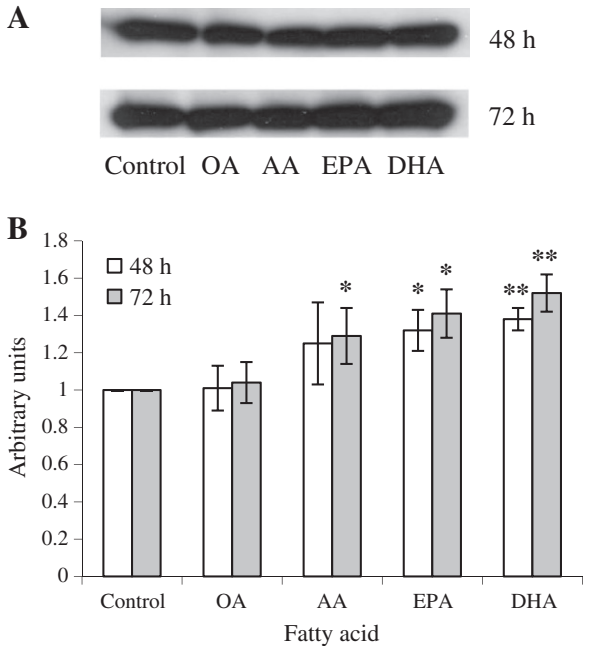

Fig. 5. Effects of PUFAs on the expression of myosin. Myosin expression was measured by Western blot analysis of the proteins extracted from the L6 cells. Cells were cultured for 48 or 72 h in DM, in the absence (control BSA) or in the presence of 20 μM fatty acid and the whole-cell protein extracts were analyzed by Western blotting with an anti-myosin antibody. (A) Representative Western blots of myosin. 10 μg of proteins were loaded in each lane. (B) Quantitation of myosin expression in Western blots by densitometric analysis. The values were normalized to tubulin levels. Myosin amounts are expressed in arbitrary units. The graph shows the means ± SE of two different experiments. *$p<0.05$ versus control.

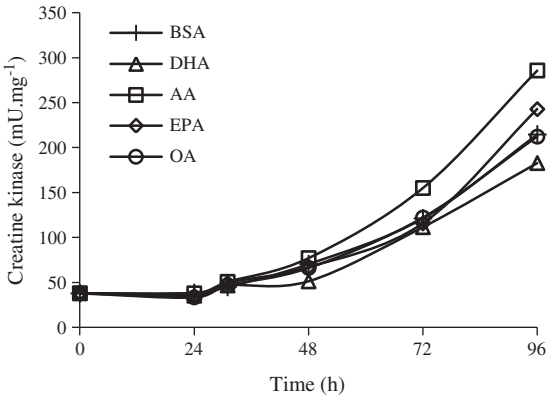

Fig. 6. Creatine kinase activity of L6 cells cultured in PUFA-supplemented differentiation medium. The L6 cells were shifted to DM in the absence (control BSA) or in the presence of 20 μM fatty acid and cultured for further 96 h before homogenization. Calculated CK activity was normalized to total protein concentration as determined by the Bradford assay.

The increased amounts of these n-3 PUFAs led to a compensatory decrease in n-6 fatty acids. It is noteworthy that the extent of AA or DHA enrichment was slightly lower in DRM than in TM, with values of 4- and 6-fold for AA, and 19- and 23-fold for DHA, respectively. Furthermore, a significant decrease in monounsaturated oleic acid (18:1 n-9) was observed under these conditions. The observed decrease in 18:1, induced by AA supplementation in PC and PE classes, as well as in PI/PS classes, was less pronounced in the DRM fractions (36, 66% and 33%, respectively) compared to TM (45, 75% and 50%, respectively). This decrease in 18:1 was also less important in DRM than in TM after DHA supplementation.

The ratio of saturated to unsaturated fatty acids was increased in TM as well as in the DRM from PUFA-treated cells when compared to control cells. Palmitic (16:0) and stearic (18:0) acid content was significantly increased in the TM and DRM lipids of AA- and DHA-supplemented cells. This likely indicates the occurrence of a mechanism compensating for the massive incorporation of PUFAs to maintain a high saturated to unsaturated ratio, typical of DRM. Cholesterol was about 5 times enriched in DRM compared to TM, but there were no significant differences in cholesterol content of DRM following PUFA treatment (data not shown). In summary, PUFAs were effectively incorporated in DRM and significantly altered the lipid environment of DRM, although to a lesser extent than with TM.

3.4. PUFA treatments modulated p70S6K1 but not Akt phosphorylation

Because PUFAs proved able to alter membrane composition and enhance the proliferation and myogenic response of L6 cells, we investigated their impact on the Akt/mTOR signalling pathway, which plays a major role in the regulation of these functions [6,27]. Akt is activated by growth factors, in particular IGFs in muscle tissue, through PI3 kinase activation that triggers its phosphorylation on crucial residues. Akt is a major activator of the mTORC1 complex, which in turn positively regulates protein synthesis and cell growth through the phosphorylation and the resulting activation of several effectors, in particular the kinase p70S6K1. The levels of phosphorylation of Akt on serine 473 and p70S6K1 on threonine 389 were evaluated over a 4-day period in differentiating L6 cells (Fig. 7 and 8 respectively). We found that a significant increase in Akt activation was induced at the onset of differentiation both in control and PUFA-treated cells. The increase in Akt phosphorylation was transient, peaking at 48 h and subsiding thereafter. Treatments by the various PUFAs did not affect the Akt activation pattern, regardless of what fatty acid had been added, demonstrating that Akt activity is not modulated by exogenous PUFAs.

Table 3
Fatty acid composition of TM and DRM lipids from control (BSA) and PUFA-treated L6 myotubes.

TM	BSA			OA			AA			DHA		
Fatty acids	PC	PI/PS	PE	PC	PI/PS	PE	PC	PI/PS	PE	PC	PI/PS	PE
14:0	6.60 ± 0.69	0.90 ± 0.01	0.53 ± 0.02	6.57 ± 0.59	1.05 ± 0.29	0.62 ± 0.17	8.10 ± 0.70	0.87 ± 0.09	0.46 ± 0.17*	6.98 ± 0.35	0.84 ± 0.04	0.41 ± 0.05
16:0	25.09 ± 1.04	8.08 ± 0.04	5.58 ± 0.22	24.77 ± 1.11	7.96 ± 1.14	5.98 ± 1.16	34.01 ± 1.19**	8.54 ± 0.28	5.95 ± 0.41	29.86 ± 0.59*	8.91 ± 0.08**	5.22 ± 0.48
18:0	7.22 ± 0.24	35.89 ± 0.54	10.62 ± 0.11	7.16 ± 0.54	35.02 ± 0.32*	9.89 ± 0.65	8.87 ± 0.32**	40.57 ± 0.80**	12.77 ± 1.29***	9.23 ± 0.06**	41.32 ± 2.35*	11.10 ± 0.73
20:0	0.12 ± 0.03	0.30 ± 0.06	0.32 ± 0.07	0.07 ± 0.03***	0.18 ± 0.06***	0.18 ± 0.10*	0.103 ± 0.02	0.25 ± 0.06	0.15 ± 0.08*	0.07 ± 0.01	0.21 ± 0.02	0.10 ± 0.02*
22:0	0.03 ± 0.03	0.22 ± 0.07	0.15 ± 0.05	0.05 ± 0.02	0.15 ± 0.01	0.09 ± 0.03*	0.07 ± 0.03	0.20 ± 0.01	0.19 ± 0.06	0.08 ± 0.01	0.15 ± 0.08	0.31 ± 0.05
24:0	0.00 ± 0.00	0.09 ± 0.03	0.02 ± 0.02	0.03 ± 0.02	0.08 ± 0.03	0.04 ± 0.03	0.03 ± 0.02	0.00 ± 0.00*	0.06 ± 0.03*	0.01 ± 0.01	0.05 ± 0.04	0.00 ± 0.00
16:1 n-9	3.98 ± 0.01	1.72 ± 0.15	0.64 ± 0.12	4.66 ± 0.16*	2.02 ± 0.18*	0.70 ± 0.16	2.79 ± 0.13***	0.99 ± 0.02*	0.22 ± 0.02*	3.15 ± 0.01***	1.35 ± 0.10*	0.32 ± 0.02*
16:1 n-7	13.58 ± 1.13	5.85 ± 0.36	7.02 ± 0.54	9.65 ± 0.22*	3.84 ± 0.39*	3.67 ± 0.30*	6.30 ± 0.30**	1.88 ± 0.11***	1.34 ± 0.74**	8.154 ± 0.41*	2.67 ± 0.18**	2.08 ± 0.08***
18:1 n-9c	20.33 ± 0.69	21.27 ± 0.58	22.20 ± 0.38	27.62 ± 2.01*	28.64 ± 0.85***	26.60 ± 0.78*	11.34 ± 0.98***	10.68 ± 0.72***	5.56 ± 0.85***	19.52 ± 0.44	17.51 ± 0.23*	10.96 ± 0.26***
18:1 n-7	15.68 ± 0.16	14.03 ± 1.03	12.08 ± 0.63	12.05 ± 0.16***	10.65 ± 0.45	8.88 ± 0.77	8.37 ± 0.06***	6.94 ± 0.22**	3.65 ± 1.03**	10.41 ± 0.34***	9.33 ± 0.55*	4.25 ± 0.34***
18:2 n-6c	0.68 ± 0.01	0.48 ± 0.02	0.73 ± 0.02	0.75 ± 0.13	0.55 ± 0.04*	0.72 ± 0.03	0.46 ± 0.08*	0.30 ± 0.12	0.19 ± 0.06***	1.23 ± 0.04***	0.49 ± 0.10	0.49 ± 0.02***
20:1 n-9	0.35 ± 0.08	0.52 ± 0.11	1.42 ± 0.00	0.73 ± 0.08	1.10 ± 0.18	2.82 ± 0.18**	0.18 ± 0.06*	0.16 ± 0.04*	0.27 ± 0.03***	0.27 ± 0.02	0.31 ± 0.02	0.28 ± 0.02***
20:2 n-6	0.21 ± 0.21	0.05 ± 0.02	0.04 ± 0.01	0.03 ± 0.01	0.09 ± 0.04	0.06 ± 0.02	0.04 ± 0.03	0.04 ± 0.02	0.05 ± 0.01	0.03 ± 0.00	0.08 ± 0.02	0.03 ± 0.00
20:3 n-6	0.18 ± 0.00	0.61 ± 0.02	0.45 ± 0.01	0.18 ± 0.04	0.55 ± 0.06	0.39 ± 0.04	0.47 ± 0.06*	0.71 ± 0.12	0.39 ± 0.04**	0.29 ± 0.01***	0.68 ± 0.11	0.28 ± 0.01***
20:4 n-6	0.51 ± 0.10	2.36 ± 0.03	5.13 ± 0.38	0.58 ± 0.15	2.25 ± 0.46	5.33 ± 0.48	12.64 ± 0.19	16.42 ± 0.33***	18.12 ± 0.86***	0.79 ± 0.01*	3.13 ± 0.17*	3.41 ± 0.17*
20:5 n-3	0.04 ± 0.04	0.06 ± 0.06	0.67 ± 0.01	0.05 ± 0.05	0.06 ± 0.04	0.63 ± 0.10	0.05 ± 0.05	0.01 ± 0.01	0.11 ± 0.06***	1.11 ± 0.04***	1.16 ± 0.12***	2.98 ± 0.00***
22:1 n-9	0.05 ± 0.05	0.24 ± 0.08	0.17 ± 0.06	0.14 ± 0.03	0.48 ± 0.13	0.38 ± 0.04	0.06 ± 0.02	0.16 ± 0.02	0.09 ± 0.03	0.06 ± 0.02	0.22 ± 0.07	0.09 ± 0.03
22:4 n-6	0.03 ± 0.01	0.25 ± 0.00	0.78 ± 0.06	0.05 ± 0.01	0.32 ± 0.02*	0.91 ± 0.07***	2.05 ± 0.02***	7.41 ± 0.25***	17.62 ± 1.03***	0.06 ± 0.01	0.24 ± 0.02	0.47 ± 0.06*
22:5 n-3	0.14 ± 0.01	0.74 ± 0.02	2.15 ± 0.07	0.14 ± 0.05	0.73 ± 0.16	2.16 ± 0.24	0.28 ± 0.03*	0.88 ± 0.04*	2.07 ± 0.62	0.32 ± 0.02***	0.95 ± 0.11	1.754 ± 0.03**
22:6 n-3	0.16 ± 0.03	0.50 ± 0.00	1.51 ± 0.09	0.16 ± 0.07	0.48 ± 0.09	1.50 ± 0.324	0.24 ± 0.03*	0.43 ± 0.05	1.17 ± 0.21*	3.81 ± 0.27***	7.24 ± 0.76***	25.36 ± 0.22***
24:1 n-9	0.06 ± 0.02	0.11 ± 0.01	0.00 ± 0.00	0.07 ± 0.02	0.20 ± 0.01	0.04 ± 0.01*	0.04 ± 0.00	0.13 ± 0.01	0.00 ± 0.00	0.04 ± 0.01	0.11 ± 0.04	0.01 ± 0.01
Saturated/unsaturated	0.70 ± 0.02	0.93 ± 0.02	0.31 ± 0.01	0.68 ± 0.07	0.86 ± 0.04*	0.31 ± 0.03	1.13 ± 0.07**	1.07 ± 0.05*	0.39 ± 0.03**	0.94 ± 0.02***	1.13 ± 0.09*	0.32 ± 0.02
n-3/n-6	0.21 ± 0.01	0.35 ± 0.01	0.61 ± 0.04	0.22 ± 0.07	0.34 ± 0.04	0.58 ± 0.03	0.04 ± 0.01***	0.05 ± 0.00***	0.09 ± 0.02**	2.18 ± 0.18***	2.02 ± 0.03***	6.43 ± 0.32***

DRM	BSA			OA			AA			DHA		
Fatty acids	PC	PI/PS	PE	PC	PI/PS	PE	PC	PI/PS	PE	PC	PI/PS	PE
14:0	7.96 ± 2.43	1.11 ± 0.29	0.95 ± 0.36	7.57 ± 2.23	1.44 ± 0.23	0.96 ± 0.08	9.64 ± 2.73	1.89 ± 0.05	0.84 ± 0.21	8.54 ± 2.96	1.71 ± 0.01	0.45 ± 0.45
16:0	38.02 ± 3.28	14.73 ± 0.44	10.62 ± 0.92	36.52 ± 1.42**	12.20 ± 0.52***	9.61 ± 0.27	47.70 ± 0.83	16.46 ± 0.70*	11.92 ± 0.34	41.245 ± 0.90	15.93 ± 0.08	10.42 ± 0.18
18:0	7.86 ± 0.59	38.86 ± 2.22	13.66 ± 0.23	7.69 ± 1.23	37.15 ± 3.28	13.23 ± 0.90	8.03 ± 0.89	36.97 ± 1.93	17.20 ± 0.39**	9.19 ± 0.97*	38.94 ± 2.19	15.15 ± 0.87
20:0	0.07 ± 0.05	0.15 ± 0.10	0.16 ± 0.11	0.02 ± 0.01*	0.21 ± 0.03	0.09 ± 0.01	0.03 ± 0.03	0.00 ± 0.00	0.16 ± 0.00	0.02 ± 0.02	0.21 ± 0.07	0.14 ± 0.05
22:0	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.04	0.02 ± 0.02	0.11 ± 0.08	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.04 ± 0.04	0.00 ± 0.00
24:0	0.01 ± 0.01	0.07 ± 0.10	0.01 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.04	0.02 ± 0.02	0.00 ± 0.00	0.38 ± 0.38	0.02 ± 0.02	0.13 ± 0.10	0.05 ± 0.05
16:1 n-9	2.53 ± 0.23	1.51 ± 0.16	0.68 ± 0.12	3.12 ± 0.82	2.45 ± 0.93	0.79 ± 0.19	1.70 ± 0.07*	1.45 ± 0.33	0.44 ± 0.15	1.92 ± 0.04*	1.49 ± 0.19	0.66 ± 0.18
16:1 n-7	7.23 ± 1.64	3.21 ± 0.69	4.04 ± 1.13	4.74 ± 1.76**	2.05 ± 0.49*	2.18 ± 1.16	3.82 ± 0.20	2.30 ± 0.52	1.08 ± 0.13	5.40 ± 0.33	2.27 ± 0.14	2.34 ± 0.15
18:1 n-9c	15.57 ± 2.47	18.18 ± 2.03	20.39 ± 3.70	24.94 ± 1.73*	26.17 ± 2.38*	27.73 ± 0.54	10.11 ± 1.13*	12.26 ± 0.27*	7.03 ± 0.59*	16.46 ± 1.08	16.70 ± 0.86	15.96 ± 0.02
18:1 n-7	12.27 ± 1.62	11.78 ± 1.52	9.56 ± 1.76	8.44 ± 3.03*	6.42 ± 2.20***	6.38 ± 2.08	7.68 ± 0.21*	7.54 ± 0.69	4.6 ± 0.11*	9.62 ± 0.55	9.79 ± 0.77	4.86 ± 0.13*
18:2 n-6c	1.38 ± 0.98	1.19 ± 0.43	1.27 ± 0.58	0.58 ± 0.05	0.87 ± 0.06	0.54 ± 0.07	0.45 ± 0.14	1.24 ± 0.02	0.53 ± 0.18	0.77 ± 0.05	0.67 ± 0.00	0.70 ± 0.05
20:1 n-9	0.33 ± 0.07	0.62 ± 0.12	1.71 ± 0.15	0.80 ± 0.24	1.71 ± 0.64	4.16 ± 1.22	0.14 ± 0.02	0.27 ± 0.12	0.44 ± 0.01***	0.24 ± 0.04	0.46 ± 0.06	0.67 ± 0.07*
20:2 n-6	0.08 ± 0.07	0.51 ± 0.48	0.68 ± 0.18	0.11 ± 0.16	0.00 ± 0.00	3.57 ± 1.74	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:3 n-6	0.13 ± 0.06	0.39 ± 0.32	0.48 ± 0.15	0.09 ± 0.02	0.29 ± 0.01	0.12 ± 0.14	0.26 ± 0.04	0.44 ± 0.04	0.32 ± 0.02	0.14 ± 0.01	0.38 ± 0.07	0.26 ± 0.03
20:4 n-6	0.26 ± 0.15	1.51 ± 0.77	4.44 ± 2.08	0.33 ± 0.26	1.18 ± 0.42	2.95 ± 0.75	5.52 ± 0.15***	8.08 ± 1.08**	15.85 ± 0.44*	0.27 ± 0.00	1.59 ± 0.30	2.823 ± 0.21
20:5 n-3	0.05 ± 0.04	0.10 ± 0.08	0.70 ± 0.32	0.03 ± 0.01	0.07 ± 0.02	0.26 ± 0.04	0.05 ± 0.01	0.00 ± 0.00	0.14 ± 0.00	0.28 ± 0.04*	0.47 ± 0.08*	1.76 ± 0.03*
22:1 n-9	0.02 ± 0.03	0.15 ± 0.03	0.17 ± 0.04	0.14 ± 0.05	0.38 ± 0.09	0.51 ± 0.10*	0.05 ± 0.05	0.26 ± 0.16	0.14 ± 0.07	0.03 ± 0.03	0.12 ± 0.12	0.07 ± 0.07
22:4 n-6	0.01 ± 0.02	0.08 ± 0.07	0.38 ± 0.16	0.04 ± 0.04	0.33 ± 0.24	0.76 ± 0.64	0.85 ± 0.02***	3.16 ± 0.28**	14.66 ± 0.72***	0.03 ± 0.01	0.19 ± 0.05	0.40 ± 0.03
22:5 n-3	0.03 ± 0.04	0.39 ± 0.17	1.60 ± 0.31	0.04 ± 0.03	0.34 ± 0.12	0.96 ± 0.16	0.12 ± 0.01	0.56 ± 0.22	1.77 ± 0.07	0.11 ± 0.00	0.48 ± 0.09	1.22 ± 0.02
22:6 n-3	0.04 ± 0.03	0.26 ± 0.06	1.01 ± 0.22	0.04 ± 0.01	0.16 ± 0.01	0.49 ± 0.10	0.04 ± 0.04	0.27 ± 0.05	0.98 ± 0.10	1.15 ± 0.12***	3.28 ± 0.32***	17.52 ± 0.28***
24:1 n-9	0.06 ± 0.08	0.14 ± 0.12	0.00 ± 0.00	0.18 ± 0.04	0.24 ± 0.11	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.12 ± 0.07	0.29 ± 0.04	0.00 ± 0.00
Saturated/unsaturated	1.35 ± 0.25	1.37 ± 0.09	0.54 ± 0.06	1.19 ± 0.12	1.20 ± 0.14	0.47 ± 0.02	2.12 ± 0.11*	1.46 ± 0.18	0.64 ± 0.00	1.63 ± 0.10	1.49 ± 0.10	0.54 ± 0.04
n-3/n-6	0.06 ± 0.06	0.21 ± 0.09	0.46 ± 0.10	0.10 ± 0.05	0.21 ± 0.05	0.21 ± 0.02	0.03 ± 0.00	0.06 ± 0.01*	0.09 ± 0.01*	1.27 ± 0.09***	1.49 ± 0.05***	4.89 ± 0.44***

Fatty acid contents were expressed in mol% and the values represent the mean ± SE from three independent determinations.
* Asterisks indicate significant differences to BSA *$p<0.05$ **$p<0.01$ ***$p<0.005$.

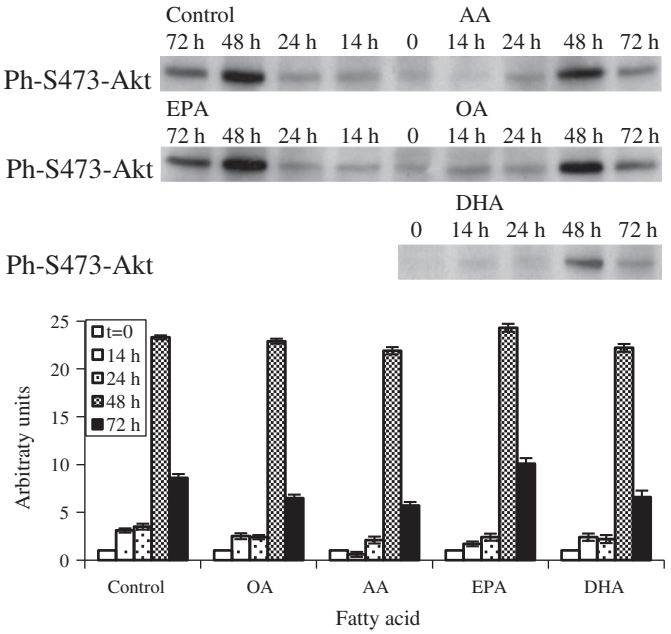

Fig. 7. Akt is transiently up-regulated during L6 cell differentiation but not affected by exogenous PUFAs. Protein extracts were prepared from L6 cells after 14, 24, 48 or 72 h of differentiation in DM in the absence (control BSA) or presence of the indicated fatty acid. After SDS-PAGE and transfer to PVDF membranes, immunoblotting was performed with a phospho-Serine 473-Akt specific antibody. (A) Representative Western blots of Akt. Note the presence of a peak of Akt phosphorylation after 48 h of differentiation whatever the treatment. Each lane contained equal amounts of total proteins. (B) The blots were quantitated by densitometric analysis, and normalized by tubulin amounts. Data represent the means ± SE of two different experiments.

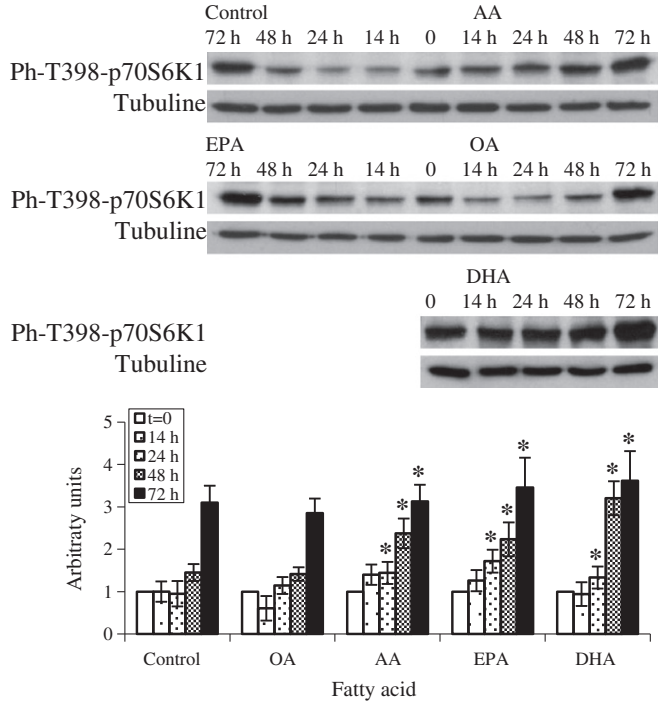

Fig. 8. p70S6K1 phosphorylation is increased during L6 cell differentiation and enhanced by exogenous PUFAs. Protein extracts were prepared from L6 cells after 14, 24, 48 or 72 h of differentiation in DM in the absence (control BSA) or presence of the indicated fatty acid. After SDS-PAGE and transfer to PVDF membranes, immunoblotting was performed with a phospho-Threonine 398-S6K1 specific antibody. (A) Representative Western blots of p70S6K. Note the clear increase in p70S6K1 phosphorylation after 48 h of differentiation in the presence of n-3 or n-6 added PUFA. Each lane contained equal amounts of total proteins. (B) The blots were quantitated by densitometric analysis, and normalized to tubulin amounts. Data represented the means ± SE of two different experiments. *$p<0.05$ versus control.

In contrast, PUFA treatments clearly affected the activation of p70S6K1, as evaluated by T389-phosphorylation (Fig. 8). The differentiation process was also accompanied by an activation of p70S6K1 in the control cells, but this started later than for Akt. Indeed, the increase in phosphorylation was only detectable at 48 h, and much more marked at 72 h. OA addition induced no significant change in this pattern, whereas the addition of n-6 and n-3 fatty acids caused an earlier and stronger activation of p70S6K1, starting at 14 h for AA and EPA and 24 h for DHA. At 48 h, the levels of phosphorylation in the presence of PUFAs were 1.5–2 times that of the control cells. A similar enhancement of the phosphorylation of the p85S6K1 isoform during treatment by PUFAs was also observed (data not shown).

4. Discussion

During the differentiation process myoblast fusion, which gives rise to multinucleated myotubes, involves the association of two distinct membranes to form a single, continuous one. Although lipid microdomain organization has been reported to be essential for normal myogenic differentiation, little is known about the redistribution of membrane lipids as well as the recruitment of new lipids that are specifically associated with myogenic differentiation. As a first issue in this paper we investigated how this myogenic differentiation altered the membrane and DRM lipid composition, and secondly we considered how modifying membrane lipid composition influenced the myogenic process.

The presence of detergent-resistant fractions in various cell types has already been demonstrated using different techniques [28–30]. Cold detergent insolubility has been adopted as the standard technique for isolating lipid domains, even though a still unsolved point is to what extent the DRM fractions retain the characteristics of the domains originally present in the membranes. For instance, changes in the protein/detergent ratio have been shown to affect the composition of isolated fractions [31]. For this reason, the isolation of DRM from L6 cells at various differentiation stages was carried out keeping these conditions constant. It is noteworthy that we could purify DRM from both L6 myoblasts and myotubes, regardless of the presence or not of caveolin-3. Although it is known that caveolin-1 and caveolin-3 are capable of driving caveolae formation, our results demonstrate that purification of DRM could be achieved, using the same detergent extraction protocol, from L6 myoblasts that only express caveolin-1 or caveolin-2, and from L6 myotubes that express the three isoforms.

The first observed alterations associated with differentiation are changes in the cholesterol to phospholipid ratio and in the saturated to unsaturated fatty acid ratio, factors which both may contribute to changes in membrane fluidity [32,33]. We observed that the CH/GPL ratio, as well as the saturated/unsaturated ratio, decreases significantly with differentiation, suggesting that membrane fluidity is necessary for membrane fusion, and might increase during this cellular process. Although the same global effect has been reported for C2 cells or the clonal line BC3H-1 [15,34], the presented analyses of total PL fatty acid composition are not as informative as the fatty acid composition of individual PL species. Striking differences in the fatty acid composition of myoblast and myotube membranes were observed in the PC, PI/PS and PE fractions. Our results show that in addition to a decrease in 16:0, 18:0, and polyunsaturated fatty acids in myotube membranes, a notable increase in n-7 and n-9 monounsaturated (16:1 n-7, 18:1 n-7 and 18:1 n-9) fatty acids was observed for the analyzed glycerophospholipids. Myogenic differentiation induces changes in several metabolic pathways, including fatty acid elongation and desaturation. Thus, the observed increase in the content of some unsaturated fatty acids (such as oleic acid) could be due to the activation of a Δ^9 desaturase.

It has been suggested that the fatty acid composition of the cultured cells mirrors the one of the medium in which they were

cultivated. The *in vitro* differentiation of myogenic cells requires a switch from a serum-rich medium to a less rich medium after the cells have reached confluence. Unlike what is used in many other studies, we induced differentiation by using the same fetal bovine serum (FBS), although at a lesser concentration in the differentiation medium. We performed the analysis of the FBS that we used, and observed that the saturated and monounsaturated FA were the most abundant (25% of 16:0, 13% of 18:0 and 19% of 18:1n-9), while PUFAs were minor components (with 3.8% for AA and 2.2% for DHA). The lipid composition of the serum was clearly different from that of TM or DRM from both myoblasts and myotubes, and it thus seems likely that the changes that accompany differentiation were due to the differentiation itself rather than to the lower serum content in the differentiation medium.

This medium also contained AVP, which was reported to stimulate phospholipases, such as phospholipase A2 (PLA2), phospholipase C (PLC) as well as phospholipase D (PLD). However, the concentrations of AVP required to induce myogenesis are only compatible with the activation of phospholipase D, whereas 100-fold higher AVP doses are required to trigger the stimulation of phospholipase C [35,36]. It is noteworthy that PLD1 expression is down-regulated as soon as the L6 cells begin to differentiate [37]. This decrease was observed in both cells differentiated by culture in low-serum medium and those differentiated in the presence of AVP, showing that the down-regulation of PLD was linked to the differentiation itself, regardless of the method for its induction. The effect of AVP on PLD activity is thus expected to have little consequence on myotube lipid composition. On the other hand, the activation of phospholipase A2 by AVP during the differentiation process could lead to a lower percentage of arachidonate in myotube compared to myoblast membranes, due to a release of arachidonate which could ultimately be metabolized into prostaglandins [38]. Future research should determine the impact of this metabolism on the lipid composition of myotube membranes.

Whereas the lipid and fatty acid composition of total membranes was significantly modified during the myogenic process, the DRMs were invariably enriched in cholesterol, sphingomyelin and saturated fatty acids. Myotube DRM contained a lower proportion of cholesterol than undifferentiated myoblasts. However, the decrease in the CH/GPL ratio was not as important as the one observed for total membranes. Furthermore, the relative enrichment in cholesterol was very similar for both preparations of DRM when compared to the heavy fractions (data not shown). Similarly to what was observed in total membranes, but to a lower extent, the analysis of the DRM fatty acyl composition of specific PL species shows a lower amount of 16:0 and 18:0 in myotubes compared to myoblasts, and also a decrease in polyunsaturated fatty acid percentages (20:5 n-3 and 22:6 n-3). These changes correlate with a decrease in cholesterol during differentiation, and could be related to the more extended structure of saturated acyl chains compared to unsaturated ones, and their ability to better pack into each other and with cholesterol [39]. The lower degree of fatty acid saturation and cholesterol content associated with differentiation would thus allow a looser packing of the lipid bilayer, and probably a lower rigidity of the membrane in myotubes as compared with myoblasts.

In addition, our results showed that as myoblast differentiation leads to a decrease in long chain PUFAs, this variation is accompanied by a modification of the n-3 to n-6 ratio in myotube membrane lipids, essentially in the PC and PE species. PUFA acyl chains are extremely flexible and can rapidly change conformational states. However, the acyl chain flexibility differs between n-3 and n-6 fatty acids and the number of double bonds significantly alters membrane fluidity [40,41]. In our study, the observed variation in the n-3 and n-6 fatty acyl composition of L6 membranes during differentiation could be related to modifications in the physical properties of the lipid membrane. Besides, it is likely that the changes in PUFA composition within the membrane phospholipids influence the distribution and activity of proteins, and thereby affect the differentiation mechanism. It was recently shown that dynamic clustering and dispersion of lipid rafts play a key role in the changes in plasma membrane composition that occur prior to membrane fusion in myogenic cells, demonstrating that lipid rafts control cell fusion in a particular mode [42]. Our study provides further insights into the lipid modifications that take place in lipid rafts during myogenic differentiation.

In vitro observations suggest that lipid rafts are sensitive to bilayer remodelling induced by manipulating the type of PUFAs provided to the cells in culture [17–19,43]. In the present study, we investigated the effect of PUFA supplementation on the L6 membrane fatty acid composition and the consequences for cell differentiation. Previous *in vitro* studies have shown stimulatory effects of various fatty acids (including linoleic acid, isomers of conjugated linolenic acid, n-3 and n-6 PUFAs) on the differentiation of rat L6, or mouse C2C12 skeletal muscle cells into myotubes [20,21]. To our knowledge, our study is the first to compare the effects of n-3 and n-6 PUFAs on the differentiation of skeletal muscle cells, including in parallel the analysis of changes in lipid composition occurring in both DRM and total membranes.

The presented results showed that PUFAs promote the morphological changes linked to myogenic differentiation, as reflected by the increased myogenic index. Differentiation of PUFA-supplemented cells resulted in an increased expression of muscle-specific proteins, such as caveolin and myosin, compared to control cells. Surprisingly, creatine kinase activity was not modified after PUFA supplementation, although it is considered a reliable marker for the degree of differentiation in the C2C12 mouse muscle cell line and primary skeletal muscle cells [44,45].

Evaluation of the cell membrane fatty acid profile revealed a significant enrichment of either AA or DHA into the membranes of L6 cells treated with the corresponding fatty acid. This is consistent with other reports demonstrating the incorporation of n-3 and n-6 PUFA into the membrane of different cell types both *in vitro* [43,46,47] and *in vivo* [10,17,48] as well as the incorporation into the whole skeletal muscle [11]. The addition of exogenous DHA (22:6 n-3) was associated with an increased incorporation of the supplemented fatty acid, and of EPA (20:5 n-3), probably originating from the retroconversion of DHA to EPA by L6 cells [46,49–51]. Since extensive membrane reorganization occurs during myoblastic differentiation (as evidenced by a decrease in PUFAs content), it was rather surprising that PUFA incorporation in total membrane enhanced myogenic differentiation. Furthermore, we also observed a significant enrichment of AA and DHA into DRM of PUFA-treated L6 myotubes with a concomitant decrease of mono-unsaturated fatty acids. Therefore, the notion that DHA is excluded from DRM because of its structural incompatibility with cholesterol was not verified in this case. In what can be viewed as a compensatory mechanism maintaining the DRM properties, fatty acid composition of L6 DRM also evidenced moderately increased levels of saturated fatty acids, compatible with the high packing of liquid-ordered raft domains [52]. PUFA supplementation not only increased the levels of the corresponding n-3 or n-6 PUFA, but also decreased n-9 fatty acids in both total membrane and DRM preparations. This could be related to a reduced expression of Δ^9 desaturase induced by PUFAs, as suggested by previous studies showing that long-chain PUFAs reduce the expression of this enzyme, with as a consequence lowered levels of monounsaturated and increased levels of saturated fatty acids [53,54].

The modulation of myogenic differentiation by PUFA supplementation can probably not be solely explained by modulation of metabolic pathways such as elongation/desaturation. It seems likely that the alteration of the fatty acyl composition of membrane lipids could directly affect the displacement of proteins from DRM

by interdigitating fatty acyl moieties [42]. In addition, PUFAs might influence the action of signalling complexes by acting as precursors for eicosanoids synthesis.

The role of Akt, a downstream effector of PI3 kinase, and p70S6K1 in cell growth and survival has been extensively studied [55]. Activation of the Akt-mTOR-p70S6K1 pathway mediates both differentiation in myoblasts and hypertrophy in myotubes [6]. Skeletal muscle differentiation clearly requires the coordination of multiple signalling pathways that regulate specific gene expression. In particular, a promyogenic role has been assigned to mitogen-activated protein kinase (MAPK) pathways, and to the PI3 kinase-Akt pathway [56,57]. Membrane translocation is a prerequisite for the full activation of Akt by enabling successive phosphorylations [58] by kinases such as PDK. It was reported that the translocation of Akt to the plasma membrane is neither efficient nor stable upon interaction with PIP3 only, but also requires the interaction with acidic phospholipids in the membrane, but not at the PIP3 binding pocket [59]. Activation and phosphorylation of Akt might thus be modulated by modifications of both the phospholipid and the fatty acid composition of the membrane. Furthermore, there is evidence in the literature that certain fatty acids can induce the activation of MAPK signalling [60,61]. In the present study, we found that exogenous PUFAs significantly increased p70S6K1 phosphorylation during differentiation, independently of changes in the phosphorylation of Akt. Akt phosphorylation occurred earlier than p70S6K1 phosphorylation during the differentiation process, and was not modified by the addition of fatty acids. These findings suggest that p70S6K1 might mediate the stimulatory action of n-3 or n-6 PUFAs on the differentiation process, possibly via its effects on protein translation and the expression of myogenic factors. The lack of significant effects of these PUFAs on Akt activation shows that the fatty acids altered neither the activity of Akt at the plasma membrane nor its translocation to the correct site. In addition, it suggests that mTORC1 and its effector p70S6K1 could be activated by fatty acids via a pathway other than the Akt pathway, possibly a MAPK pathway.

In conclusion, our results showed that n-3 as well as n-6 PUFAs presented a marked activating effect on the myogenic differentiation of L6 cells, based on an increased myogenic index and the expression of caveolin and myosin. In parallel, these PUFAs profoundly altered membrane and DRM lipid composition. These results support the hypothesis that fatty acyl composition of total membrane and DRM influence signalling complexes involved in myogenic differentiation. The mechanism by which PUFAs activate the mTORC1/p70S6K1 signalling pathway and whether this signal is the cause of enhanced myogenic response remain to be investigated.

Acknowledgements

We are very grateful to V. Fitzpatrick and W. Lesniak for correcting the English.

References

[1] R.L.S. Perry, M.A. Rudnicki, Molecular mechanisms regulating myogenic determination and differentiation, Front. Biosci. 5 (2000) D750–D767.
[2] V. Andres, K. Walsh, Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis, J. Cell Biol. 132 (1996) 657–666.
[3] S.B.P. Charge, M.A. Rudnicki, Cellular and molecular regulation of muscle regeneration, Physiol. Rev. 84 (2004) 209–238.
[4] Y.Q. Li, B.H. Jiang, W.Y. Ensign, P.K. Vogt, J.H. Han, Myogenic differentiation requires signalling through both phosphatidylinositol 3-kinase and p38 MAP kinase, Cell. Signal. 12 (2000) 751–757.
[5] Z.G. Wu, P.J. Woodring, K.S. Bhakta, K. Tamura, F. Wen, J.R. Feramisco, M. Karin, J.Y.J. Wang, P.L. Puri, p38 and extracellular signal-regulated kinases regulate the myogenic program at multiple steps, Mol. Cell. Biol. 20 (2000) 3951–3964.
[6] S.C. Bodine, T.N. Stitt, M. Gonzalez, W.O. Kline, G.L. Stover, R. Bauerlein, E. Zlotchenko, A. Scrimgeour, J.C. Lawrence, D.J. Glass, G.D. Yancopoulos, Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo, Nat. Cell Biol. 3 (2001) 1014–1019.
[7] K. Simons, E. Ikonen, Functional rafts in cell membranes, Nature 387 (1997) 569–572.
[8] F.R. Maxfield, Plasma membrane microdomains, Curr. Opin. Cell Biol. 14 (2002) 483–487.
[9] D.A. Brown, E. London, Structure and function of sphingolipid- and cholesterol-rich membrane rafts, J. Biol. Chem. 275 (2000) 17221–17224.
[10] S. Liu, V.E. Baracos, H.A. Quinney, M.T. Clandinin, Dietary omega-3 and polyunsaturated fatty-acids modify fatty acyl composition and insulin binding in skeletal muscle sarcolemma, Biochem. J. 299 (1994) 831–837.
[11] K.D. Stark, S.Y. Lim, N. Salem, Docosahexaenoic acid and n-6 docosapentaenoic acid supplementation alter rat skeletal muscle fatty acid composition, Lipids Health Dis. 6 (2007) 11.
[12] S.K. Abbott, P.L. Else, A.J. Hulbert, Membrane fatty acid composition of rat skeletal muscle is most responsive to the balance of dietary n-3 and n-6 PUFA, Br. J. Nutr. 103 (2010) 522–529.
[13] G. Schmitz, J. Ecker, The opposing effects of n-3 and n-6 fatty acids, Prog. Lipid Res. 47 (2008) 147–155.
[14] S.R. Wassall, W. Stillwell, Polyunsaturated fatty acid-cholesterol interactions: domain formation in membranes, Biochim. Biophys. Acta 1788 (2009) 24–32.
[15] M. Nakanishi, E. Hirayama, J. Kim, Characterisation of myogenic cell membrane: II. Dynamic changes in membrane lipids during the differentiation of mouse C2 myoblast cells, Cell Biol. Int. 25 (2001) 971–979.
[16] M. Borkman, L.H. Storlien, D.A. Pan, A.B. Jenkins, D.J. Chisholm, L.V. Campbell, The relation between insulin sensitivity and the fatty-acid composition of skeletal-muscle phospholipids, N. Engl. J. Med. 328 (1993) 238–244.
[17] D.W.L. Ma, J.M. Seo, L.A. Davidson, E.S. Callaway, Y.Y. Fan, J.R. Lupton, R.S. Chapkin, n-3 PUFA Alter caveolae lipid composition and resident protein localization in mouse colon, FASEB J. 18 (2004) 1040–1042.
[18] O. Diaz, A. Berquand, M. Dubois, S. Di Agostino, C. Sette, S. Bourgoin, M. Lagarde, G. Nemoz, A.F. Prigent, The mechanism of docosahexaenoic acid-induced phospholipase D activation in human lymphocytes involves exclusion of the enzyme from lipid rafts, J. Biol. Chem. 277 (2002) 39368–39378.
[19] V. De Smedt-Peyrusse, F. Sargueil, A. Moranis, H. Harizi, S. Mongrand, S. Laye, Docosahexaenoic acid prevents lipopolysaccharide-induced cytokine production in microglial cells by inhibiting lipopolysaccharide receptor presentation but not its membrane subdomain localization, J. Neurochem. 105 (2008) 296–307.
[20] M.S. Hurley, C. Flux, A.M. Salter, J.M. Brameld, Effects of fatty acids on skeletal muscle cell differentiation *in vitro*, Br. J. Nutr. 95 (2006) 623–630.
[21] J.H. Lee, H. Tachibana, Y. Morinaga, Y. Fujimura, K. Yamada, Modulation of proliferation and differentiation of C2C12 skeletal muscle cells by fatty acids, Life Sci. 84 (2009) 415–420.
[22] Q.R. Li, M. Wang, L. Tan, C. Wang, J. Ma, N. Li, Y.S. Li, G.W. Xu, J.S. Li, Docosahexaenoic acid changes lipid composition and interleukin-2 receptor signalling in membrane rafts, J. Lipid Res. 46 (2005) 1904–1913.
[23] C. Nervi, L. Benedetti, A. Minasi, M. Molinaro, S. Adamo, Arginine-vasopressin induces differentiation of skeletal myogenic cells and up-regulation of myogenin and myf-5, Cell Growth Differ. 6 (1995) 81–89.
[24] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[25] J.C.M. Stewart, Colorimetric determination of phospholipids with ammonium ferrothiocyanate, Anal. Biochem. 104 (1980) 10–14.
[26] L.J. Macala, R.K. Yu, S. Ando, Analysis of brain lipids by high-performance thin-layer chromatography and densitometry, J. Lipid Res. 24 (1983) 1243–1250.
[27] M. Laplante, D.M. Sabatini, mTOR signaling at a glance, J. Cell Sci. 122 (2009) 3589–3594.
[28] A. Draeger, K. Monastyrskaya, F.C. Burkhard, A.M. Wobus, S.E. Moss, E.B. Babiychuk, Membrane segregation and downregulation of raft markers during sarcolemmal differentiation in skeletal muscle cells, Dev. Biol. 262 (2003) 324–334.
[29] G.M. Smythe, J.C. Eby, M.H. Disatnik, T.A. Rando, A caveolin-3 mutant that causes limb girdle muscular dystrophy type 1C disrupts Src localization and activity and induces apoptosis in skeletal myotubes, J. Cell Sci. 116 (2003) 4739–4749.
[30] C. Pato, F. Stetzkowski-Marden, K. Gaus, M. Recouvreur, A. Cartaud, J. Cartaud, Role of lipid rafts in agrin-elicited acetylcholine receptor clustering, Chem. Biol. Interact. 175 (2008) 64–67.
[31] E.B. Babiychuk, A. Draeger, Biochemical characterization of detergent-resistant membranes: a systematic approach, Biochem. J. 397 (2006) 407–416.
[32] D. Stubbs, A.D. Smith, The modification of mammalian membrane poly-unsaturated fatty-acid composition in relation to membrane fluidity and function, Biochim. Biophys. Acta 779 (1984) 89–137.
[33] W.C. Hung, M.T. Lee, F.Y. Chen, H.W. Huang, The condensing effect of cholesterol in lipid bilayers, Biophys. J. 92 (2007) 3960–3967.
[34] M.F. Pediconi, L.E. Politi, C.B. Bouzat, E.B. Delossantos, F.J. Barrantes, Myogenic differentiation of the muscle clonal cell-line BC3H-1 is accompanied by changes in its lipid-composition, Lipids 27 (1992) 669–675.
[35] A. Teti, F. Naro, M. Molinaro, S. Adamo, Transduction or arginine-vasopressin signal in skeletal myogenic cells, Am. J. Physiol. 265 (1993) C113–C121.
[36] F. Naro, V. Donchenko, S. Minotti, L. Zolla, M. Molinaro, S. Adamo, Role of phospholipase C and D signalling pathways in vasopressin-dependent myogenic differentiation, J. Cell. Physiol. 171 (1997) 34–42.
[37] H. Komati, F. Naro, S. Mebarek, V. De Arcangelis, S. Adamo, M. Lagarde, A.F. Prigent, G. Nemoz, Phospholipase D is involved in myogenic differentiation

through remodeling of actin cytoskeleton, Mol. Biol. Cell 16 (2005) 1232–1244.
[38] F. Naro, V. De Arcangelis, C. Sette, C. Ambrosio, H. Komati, M. Molinaro, S. Adamo, G. Nemoz, A bimodal modulation of the cAMP pathway is involved in the control of myogenic differentiation in L6 cells, J. Biol. Chem. 278 (2003) 49308–49315.
[39] S.R. Shaikh, A.C. Dumaual, A. Castillo, D. LoCascio, R.A. Siddiqui, W. Stillwell, S.R. Wassall, Oleic and docosahexaenoic acid differentially phase separate from lipid raft molecules: a comparative NMR, DSC, AFM, and detergent extraction study, Biophys. J. 87 (2004) 1752–1766.
[40] N.V. Eldho, S.E. Feller, S. Tristram-Nagle, I.V. Polozov, K. Gawrisch, Polyunsaturated docosahexaenoic vs docosapentaenoic acid — differences in lipid matrix properties from the loss of one double bond, J. Am. Chem. Soc. 125 (2003) 6409–6421.
[41] K. Rajamoorthi, H.I. Petrache, T.J. McIntosh, M.F. Brown, Packing and viscoelasticity of polyunsaturated omega-3 and omega-6 lipid bilayers as seen by H-2 NMR and X-ray diffraction, J. Am. Chem. Soc. 127 (2005) 1576–1588.
[42] A. Mukai, T. Kurisaki, S.B. Sato, T. Kobayashi, G. Kondoh, N. Hashimoto, Dynamic clustering and dispersion of lipid rafts contribute to fusion competence of myogenic cells, Exp. Cell Res. 315 (2009) 3052–3063.
[43] T.M. Stulnig, J. Huber, N. Leitinger, E.M. Imre, P. Angelisova, P. Nowotny, W. Waldhaus, Polyunsaturated eicosapentaenoic acid displaces proteins from membrane rafts by altering raft lipid composition, J. Biol. Chem. 276 (2001) 37335–37340.
[44] C.F.M. Prinsen, J.H. Veerkamp, Transfection of L6 myoblasts with adipocyte fatty acid-binding protein cDNA does not affect fatty acid uptake but disturbs lipid metabolism and fusion, Biochem. J. 329 (1998) 265–273.
[45] G.L. Portier, A. Benders, A. Oosterhof, J.H. Veerkamp, T.H. van Kuppevelt, Differentiation markers of mouse C2C12 and rat L-6 myogenic cell lines and the effect of the differentiation medium, In Vitro Cell. Dev. Biol. Anim. 35 (1999) 219–227.
[46] G. Champeil-Potokar, C. Chaumontet, P. Guesnet, M. Lavialle, I. Denis, Docosahexaenoic acid (22: 6n–3) enrichment of membrane phospholipids increases gap junction coupling capacity in cultured astrocytes, Eur. J. Neurosci. 24 (2006) 3084–3090.
[47] B. Langelier, A. Linard, C. Bordat, M. Lavialle, C. Heberden, Long chain-polyunsaturated fatty adds modulate membrane phospholipid composition and protein localization in lipid rafts of neural stem cell cultures, J. Cell. Biochem. 110 (2010) 1356–1364.
[48] S. Nagahuedi, J.T. Popesku, V.L. Trudeau, J.M. Weber, Mimicking the natural doping of migrant sandpipers in sedentary quails: effects of dietary n-3 fatty acids on muscle membranes and PPAR expression, J. Exp. Biol. 212 (2009) 1106–1114.
[49] M.D. Rosenthal, M.C. Garcia, M.R. Jones, H. Sprecher, Retroconversion and delta-4 desaturation of docosatetraenoate (22-4(n-6)) and docosapentaenoate (22-5(n-3)) by human-cells in culture, Biochim. Biophys. Acta 1083 (1991) 29–36.
[50] E.R. Brown, P.V. Subbaiah, Differential-effects of eicosapentaenoic acid and docosahexaenoic acid on human skin fibroblasts, Lipids 29 (1994) 825–829.
[51] D.R. Tocher, J.R. Dick, Effects of essential fatty acid deficiency and supplementation with docosahexaenoic acid (DNA; 22: 6n–3) on cellular fatty acid compositions and fatty acyl desaturation in a cell culture model, Prostaglandins Leukot. Essent. Fatty Acids 64 (2001) 11–22.
[52] K. Simons, W.L.C. Vaz, Model systems, lipid rafts, and cell membranes, Annu. Rev. Biophys. Biomol. Struct. 33 (2004) 269–295.
[53] J.M. Ntambi, Regulation of stearoyl-CoA desaturase by polyunsaturated fatty acids and cholesterol, J. Lipid Res. 40 (1999) 1549–1558.
[54] M.T. Nakamura, T.Y. Nara, Gene regulation of mammalian desaturases, Biochem. Soc. Trans. 30 (2002) 1076–1079.
[55] D.J. Glass, Molecular mechanisms modulating muscle mass, Trends Mol. Med. 9 (2003) 344–350.
[56] J. Tureckova, E.M. Wilson, J.L. Cappalonga, P. Rotwein, Insulin-like growth factor-mediated muscle differentiation — collaboration between phosphatidylinositol 3-kinase-Akt-signaling pathways and myogenin, J. Biol. Chem. 276 (2001) 39264–39270.
[57] I. Gonzalez, G. Tripathi, E.J. Carter, L.J. Cobb, D.A.M. Salih, F.A. Lovett, C. Holding, J.M. Pell, Akt2, a novel functional link between p38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways in myogenesis, Mol. Cell. Biol. 24 (2004) 3607–3622.
[58] J.N. Kanfer, The base-exchange enzymes and phospholipase-D of mammalian tissue, Can. J. Biochem. 58 (1980) 1370–1380.
[59] C.C. Thomas, M. Deak, D.R. Alessi, D.M.F. van Aalten, High-resolution structure of the pleckstrin homology domain of protein kinase B/Akt bound to phosphatidylinositol (3,4,5)-trisphosphate, Curr. Biol. 12 (2002) 1256–1262.
[60] A.A. Gingras, P.J. White, P.Y. Chouinard, P. Julien, T.A. Davis, L. Dombrowski, Y. Couture, P. Dubreuil, A. Myre, K. Bergeron, A. Marette, M.C. Thivierge, Long-chain omega-3 fatty acids regulate bovine whole-body protein metabolism by promoting muscle insulin signalling to the Akt-mTOR-S6K1 pathway and insulin sensitivity, J. Physiol. 579 (2007) 269–284.
[61] C. Le Foll, C. Corporeau, V. Le Guen, J.P. Gouygou, J.P. Berge, J. Delarue, Long-chain n-3 polyunsaturated fatty acids dissociate phosphorylation of Akt from phosphatidylinositol 3′-kinase activity in rats, Am. J. Physiol. 292 (2007) E1223–E1230.